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Alloplastic Implant in Primates: Culture Medium 199 -- a Sequential Histopathologic Study

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ALLOPLASTIC IMPLANT IN PRIMATES: CULTURE MEDIUM 199 -- A SEQUENTIAL HISTOPATHOLOGIC STUDY

by

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DEDICATION

To my parents, Peter and Margaret Haupers, for their love, guidance and encouragement throughout my life and education.

To my young bride, Barbara, for her love, confidence, friendship and understanding of my pursuit of this education.

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CHAPTER I

INTRODUCTION

Periodontal disease involves an inflammatory process which leads to the loss of tooth-supporting alveolar bone. It is fairly commonplace in treatment at this time to enter the affected areas surgically and by subtractive procedures bring the osseous structures to a more physiologic contour. Unfortunately, this modality of treatment can cause additional loss of alveolar bone, thus potentially weakening already weak supporting structures; or in certain areas affect the postsurgical aesthetic appearance.

As periodontal disease progresses the osseous defects become more difficult to treat by the subtractive measures. Many times these osseous defects can lend themselves to treatment by osseous grafting procedures. The ideal graft material should be acceptable to the tissues, predictable, clinically feasible and provide minimal risk to the patient.

Viable bone itself has been used as a graft material. Researchers have used cortical autografts,[1] bone blend,[2] cancellous bone and marrow grafts(intraoral and iliac

crest),[3,4] and variously prepared iliac allografts.[5,6] Some of these materials are less than ideal since they do not satisfy the practical requirements of availability in adequate amounts, ease of manipulation, minimal trauma to the patient, economy and predictability.

Indeed, researchers have been looking for a simple, reliable method and/or material which could improve our treatment of the periodontal osseous defect. Many investigators have experimented with such alloplasts (bone substitutes) as polyvinyl sponge,[7] calcium salts,[8] glass,[9] ceramics,[10] polyurethane derivatives,[11] absorbable gelatin sponge(Gelfoam) with penicillin added,[12] chondroitin sulfate,[13] various plastics and acrylics,[14] alcoholic and acidic extracts of bone and periosteum,[7] soft tissues such as urinary mucosa,[8] cartilage,[14] antireticulotoxic sera,[15] total embryonic extracts[15] and metals.[14] Several investigators have felt that plaster of Paris has some merit in treating osseous defects.[16,17,18] None of these materials studied has been universally successful in achieving the physiologic goals of a graft material.

For years tissue culture medium has been used as a substrate for growth of cells <u>in vitro</u>. Many of the

constituent nutrients of tissue culture medium can act to stimulate growth and act as a substrate for cells preparing for mitosis. Tissue culture medium has been used to store several bone marrow implants for short periods of time.[5] It has also been used as a suspending medium prior and subsequent to bone freezing storage techniques.[19] Toto, et al. used tissue culture in mice in vivo and demonstrated cellular support and probably cellular stimulated growth.[20]

Since tissue culture medium has such encouraging results in vitro and has been found to be acceptable to tissues in vivo, it would be desirable to question whether a medium could support and stimulate growth of cells in vivo in periodontal additive osseous surgical procedures. An investigation was designed to study the regenerative, supportive, and nutritive capabilities of tissue culture medium as an alloplastic osseous implant material. The purpose of this investigation is to examine histologically the sequential healing phenomenon of surgically created two-walled osseous defects in the rhesus monkey using Culture Medium 199* as an alloplastic implant material.

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*Medium 199, Cat. No. E-12, Grand Island Biological Co. Grand Island, New York.

CHAPTER II

REVIEW OF THE LITERATURE

Creating a cellular environment favorable to bone maintenance and growth could be a favorable asset in osseous regenerative periodontal surgical procedures. Of interest in the maintenance and growth of bone are the many *in vitro* studies related to culture media. As early as 1910, *in vitro* cultivation of bone was described by Carrel and Burrows;[21] who, in that point of the literature, described an outwandering of cells from the explant but ossification was not noted in the new tissue.

Later in 1928, Fell,[22] and Robinson(1929)[23] were able to show osteogenesis in cultures *in vitro* with embryonic limb cartilage. Friedheim(1930)[24] stated that bone will develop in cultures of perichondrium obtained from the limbs of rat embryos. Classic early literature of *in vitro* osteogenesis must also include the work of Fell in 1931[25] and 1932[26] which delt with an osteogenic capacity *in vitro* of the embryonic periosteum of chick limb bone.

Jacobson and Fell(1941)[27] made an important observation in relation to osteogenesis in tissue culture. This

work is of great importance since it showed categorically that although chondrogenesis and osteogenesis are often closely related, they can also occur as distinctly independent processes.

Diffusion chamber techniques were developed to definitely establish whether or not an implant material is capable of further growth and differentiation. A chamber technique first utilized by Algire et al. (1954),[28] permitted diffusion of nutrients and metabolites through a porous filter between host tissue and implant, but prevented the passage of cells. Later, Algire(1957)[29] referred to this technique as a form of in vivo tissue culture, thus establishing a methodology for determination of implant growth and differentiation capabilities.

The ability of bone survival and actual proliferation was shown well by Goldhaber in 1958.[30] Goldhaber demonstrated in mice that cells of bone isografts implanted subcutaneously within diffusion chambers survived, and, interestingly, actually had an ability for cell proliferation with new bone formation. Later, Goldhaber(1961)[31] immunized the host mice homograft tissues prior to subcutaneous implantation of homograft bone within diffusion chambers and this immunization did not prevent new bone

formation on the host side of the filter. Thus, suggesting that its origin from host cells was in response to a diffusible osteogenic inductor from within the chamber rather than from undetected escaping homograft cells. Goldhaber further demonstrated bone induction across a millipore filter *in vivo*[32] stating that the findings support the fact that a new, vital bone found on the host side of the diffusion chamber was derived from host tissue in response to a diffusible osteogenic inductor coming from the new homograft bone laid down on the inner aspect of the chamber filter.

Bone induction was discussed by Urist[33] who termed the "bone induction principle" stating that a tissue substrate may exert a physio-chemical effect upon competent mesenchymal cells to stimulate their differentiation into osteoblasts capable of both osteogenesis and further induction(autoinduction). Thus, local cell environment may be a determining factor in bone maintenance and growth.

In reviewing an implant material attention must be focused on osteogenic activity. Considering osteogenesis it may be worth reviewing concepts of cell types accepted by the literature. Four major cell types are recognized in bone: osteoblasts, osteocytes, osteoclasts and mesen-

chymal cells.[34] Osteoblasts have been suggested to arise from three possible origins: Burns[35] suggested that they arise from pre-existing osteoblasts, Ham and Harris[36] suggested that they arise from the endosteal lining of the marrow cavities and Bloom suggested they arise from the previous two sources plus from the perivascular undifferentiated mesenchymal cells. The term "osteoblasts" is used to designate those cells that lie in close contact with the bone matrix surface representing bone lining cells.[38] Osteocytes represent those cells surrounded by mineralized bone and communicate with the vasuclar system by a series of canaliculi through which osteocyte and osteoblast processes communicate. The osteocyte occupies a lacunar space and is surrounded by a capsule of nonmineralized osteoid. The osteocyte in its relationship to the intercellular matrix is engaged not only in the maintenance of open pathways in bone but also in a fluid transport mechanism.[39] Also, the periosteocyte capsule has been implicated as having a potential transport function.[40] Since the fine structure of the osteocyte resembles the osteoblast, it is suggested that both are involved in active cell functions of protein and polysaccharide synthesis, resorption, and mineral deposition and release.[38]

The osteoclast is equipped for catabolic activity.[38] It is a multinucleated cell with phagocytic vacuoles, intracellular canaliculi, numerous large mitochondria and lysosomes. It is likely that the osteoclasts are involved in bone remodelling activity and contribute mineral to the plasma in the process of bone resorption.

The last cell type recognized in bone is the pluripotential mesenchymal cell that can contribute to the bone-forming or bone-resorbing cell population.

Within the periodontal literature scant use of culture media is found. Primarily the only cited use of an essential culture medium was by using the medium for short term storage of bone marrow being used for grafting providing the nutritional needs of the nutritional needs of the bone cells,[5] and as a suspending nutrient medium prior and subsequent to bone freezing storage techniques.[19]

The literature supplies vast support that nutritive substances can act to stimulate growth and can act as a substrate for cells preparing for mitosis. Tissue culture medium serves as a substrate for growth of cells *in vitro*. It has been shown to be useful for bone storage in periodontal procedures. These are *in vitro* studies.

The *in vivo* use of tissue culture medium has shown

cellular support and probably cellular stimulated growth in mice(Toto et al., 1968).[20]

Infusion solutions containing the essential amino acids, glucose, vitamins, trace elements, fat emulsions and electrolytes are being used routinely for patients on complete intravenous nutrition.[41] The in vivo use of nutrients is not a new idea. For years parenteral and alimentary nutrition have sustained many patients for long periods of time. This was first suggested by Sir Christopher Wren, who predicted in 1659 the possibility of introducing any liquid immediately into the blood stream.[42] Lidstrom records the early work of Bidder and Schmidt(1852) and Voit(1866) both of whom showed that nitrogen balance could be influenced by the administration or withholding of protein.[43] It was not until 1944 that Wretlind produced the first really satisfactory amino acid preparation for intravenous use by means of enzymatic hydrolysis of casein and subsequent dialysis.[44] Sustaining life by use of intravenous and alimentary feeding is evidence supporting the use of basic nutrients in vivo but the most significant information concerning the use of nutrient fluids is that intravenous feeding has been shown to support growth in a child and has helped restore weight loss in adults suggest-

ing the body utilizes these nutrients effectively.[45]

Since the actual material used for intravenous feeding is quite similar to many culture media systems (see Appendices I and II) and the human body has been shown to effectively utilize these basic nutrients through the intravenous route, perhaps the use of specific nutrients placed in osseous defects could be utilized by the tissues present for growth and repair of these defects.

Since tissue culture medium had many encouraging results *in vitro*, had been found acceptable to tissues *in vivo*, and since similar solutions can support growth intravenously, it may be desirable to question whether a medium could support and stimulate growth of cells *in vivo* in periodontal additive osseous surgical procedures.

Paul(1959)[46] has described that for a medium to be utilized it must possess certain properties and be in balance for cellular support. Components essential for immediate survival of cells and tissues have been precisely defined. It is essential to control osmotic pressure and pH, while a source of energy and certain inorganic ions are necessary for all but the briefest survival. These requirements are met by a combination of salts and glucose. A simple medium of this sort is referred to as a "balanced

salt solution". These salt solutions are the basis of all culture media except the very simplest of natural media.

Cells and tissues will survive for a brief time in a balanced salt solution but for more prolonged survival other factors are necessary. These factors, normally present in whole serum, can be replaced by a synthetic mixture. For survival for long periods of time mammalian cells require, in addition to a balanced salt solution, all the essential amino acids, oxygen, vitamins and serum protein. At the present, growth in the complete absence of serum requires in addition to those factors stated above, all the other common amino acids, co-enzymes, other vitamins and nucleosides.

The preparation and constituents of essential medium was presented well by Paul(1959).[46] In consideration of osseous maintenance and growth the constituents of Culture Medium 199 will be examined.

Collagen is the principal organic component of bone and is made up of amino acids.[47] Collagen is a fibrous protein and an essential component of connective tissue found in bone.[48] Collagen has a very unique amino acid composition, the most striking feature of which is the fact that one-third is the combination of proline, hydroxypro-

line, and alanine and another one-third of the amino acid comprise glycine.[49] In structural terms, the fact that glycine makes up one-third of the amino acid residues is significant. In order to enable the polypeptide chain to assume the helical conformation it is necessary that the small glycine residue should occupy every third position.[50] The most direct precursory role of amino acids is that involving protein synthesis. Studies by Eagle et al.(1961) showed that a critical threshold level of intracellular amino acid concentration exists to initiate cell growth and protein synthesis.[51] It is also interesting to mention that cultured cells contain a large pool of free amino acids in dynamic equilibrium with the surrounding fluid. When these amino acids are provided they are concentrated within the cell to reach levels three to fifty times that in the medium.[52] An interesting finding related to the amino acid guanine, is the fact that guanine had induced intra-muscular osteogenesis.[53]

A major advance in animal nutrition which led to new thinking about nutrition at the cell level was represented by the demonstration that rats could be maintained in nitrogen balance if the protein of the diet was replaced by a mixture of the component amino acids.[54]

In the synthesis of collagen, there are two unusual amino acids, hydroxyproline and hydroxylysine. These amino acids are created by the addition of hydroxyl(OH) groups to some of the proline and lysine units in the previously formed amino acid chain.[55] Ascorbic acid is probably involved in both the hydroxylation of proline to hydroxyproline and lysine to hydroxylysine.[56] Jeney and Toro[57] when growing fibroblasts in tissue culture medium demonstrated a more rapid production with the addition of vitamin C. Experiments in tissue culture by a mouse fibroblast line showed that ascorbic acid deficiency resulted in half the amount of hydroxyproline containing material being laid down; thus, supporting the hypothesis that ascorbic acid affects the intermolecular cross-linking of collagen through certain hydroxylysine residues as well as affecting the hydroxylation of proline.[58] Also, ascorbic acid promoted collagen synthesis in isolated bone cells by directly stimulating the hydroxylation of a proline-rich peptide.[59] Vitamin C has been suggested to play an important role in the production of an inflammatory reaction to injury of bone in the organization of the blood clot resulting therefrom and in the formation of the ground substance of trabeculae.[60]

It had been demonstrated in vivo that the amount of calcification may vary directly with the amount of vitamin C in the diet suggesting that vitamin C does play a role in the process of calcification.[61]

Vitamin D may exert a local role in calcification. It had been demonstrated that vitamin D may aid in maintaining the calcium and phosphate concentration of plasma at supersaturated levels in regard to bone mineral which in turn is necessary for normal calcification in bone.[62] De Luca in 1967 suggested that vitamin D acts by facilitating the transport of calcium from bone fluid to bone cell.[63] The overall effect of vitamin D is to maintain calcification.[64]

Vitamin A has specific effects on bone, not only for growth and maintenance of normal structure but has effect in excess.[65] Vitamin A accelerated the histologic sequence of endochondral ossification and in particular of bony remodelling in many situations.[66]

Glucose has been demonstrated to be of value in culture media in support of bone.[67] In absence of glucose chick bone rapidly degenerated. Sucrose substituted for glucose in the synthetic media resulted in resorption of the matrix.[67]

McLean and Bloom(1940)[69] concluded that the bone matrix may be calcifiable as soon as the tissue is recognizable as bone, and that the delay sometimes observed in the deposit of mineral may be ascribed to a lag in the supply of the necessary minerals. Neuman and Neuman (1958)[70] demonstrated that the blood serum must be supersaturated with the essentials of bone mineral if calcification is to occur.

There are two theories of calcium salt formation. One states that a high enough local ionic concentration of calcium and phosphate somehow must accumulate for precipitation to occur. The other states that some organic component, collagen in the case of bone, has properties mimicking those of apatite crystals. Extracellular fluid is supersaturated and metastable with respect to calcium and phosphate, such an organic structure could act as a seed crystal and thus as a starting point for a self-propagating process.[71]

Murry stated that there is an affinity of the fibrin clot for calcium and may thus aid in the retention of bone powder.[72] Also, Murry noted that calcium in the graft is necessary for the calcification of newly forming bone and cementum.

Wadkins has shown that exposure of an ordinarily noncalcifying matrix in vivo to an unphysiologically high concentration of calcium can produce a matrix which catalyzed mineral formation.[73] That mineralization was more dependent on calcium ion than phosphate ion concentration was illustrated in culture media mineralization of chick tibias.[74] It has been demonstrated that the process of bone surface exchange of calcium takes place in a layer of bone one to four microns thick[75] and that calcium is the first ion to be found.[76]

Hadhazy has demonstrated that Na_2HPO_4, and $CaCl_2$ can have osteogenic induction properties when placed intramuscularly.[77]

Magnesium ion is a necessary cofactor for several biochemical reactions, especially, transphosphorylation reactions such as transfer of phosphate to and from the storage molecules ADP and ATP. Rubin(1975) found in culture media that decreased availability of Mg^{++} led to a coordinate deceleration in cell function and that increased availability led to an acceleration. That an inorganic ion might control growth and differentiation is not a totally new idea. But the Mg^{++} theory is sufficiently different from other models of cellular

control.[78]

The aforementioned investigations show promising information toward cellular support, growth and induction as related to tissue culture media. Various constituents within the medium have demonstrated some supportive and beneficial qualities. Thus, this study was designed to evaluate the capacities of tissue culture medium used as an alloplastic implant material in periodontal defects. The investigation technique had been repeatedly utilized in the research of osseous defects. Surgically created defects were created in the alveolar bone of rhesus monkeys as previously established by Coverly, Skinner, Poulsom, Jenkins and others.[79,80,81,82] The purpose of this investigation is to evaluate the response of alloplastic implants of Medium 199 in surgically created osseous defects in rhesus monkeys.

CHAPTER III

MATERIALS AND METHODS

A. EXPERIMENTAL DESIGN

Two adult male rhesus monkeys(Macaca mulatta) were utilized as experimental models in this study. The experiment covered a one-hundred and seven day interval utilizing the first twenty-one days as a quarantine and tuberculosis inoculation period. This period allows the monkeys to acclimate to the Loyola animal care facility and a definite parameter of health to be established. Throughout the experimental period, the animals maintained their physical parameters as recorded during their quarantine, and appeared to remain in good health during the remaining eighty-six days of the experimental period.

The animals demonstrated a slight marginal gingivitis with varying amounts of materia alba, plaque and calculus. The gingiva was firm in consistency and pink in color. The sulcus depths were examined and noted to be within acceptable limits(Figure 1).

The maxillary and mandibular quadrants were utilized as experimental sites. Thus, four maxillary and four

mandibular quadrants were included in the study. Two, two-walled surgically created osseous defects per quadrant were created. Created defects distal to the second premolars were utilized as the control area, and the created defects destal to the first premolar were the experimental sites used for the medium. The control site preparation and correction(by surgical curettage without the placement of culture medium implants) was accomplished at the same time as the preparation and correction of the analogous implanted defects within the same quadrant.

The one-hundred and seven day schedule of the experiment was planned to allow for a 21 day quarantine and an 86 day experimental period. This allowed the two-walled osseous defects to be created thirty days before the initial implantation. The predetermined schedule was so designed to allow the sacrifice of the control and the culture medium implants on 0, 3, 7, 14, 21, 28, 42, and 56 days, postoperatively. Each time sequence allowed for a control defect and a defect to be corrected by culture medium.

Twenty minutes prior to the surgical procedures, the monkey received an intramuscular injection of 8 mg.

Serylan* for sedation. When long procedures were encountered, an additional dose of 5 mg. Serylan was given intramuscularly as needed for sedation. Prior to the surgical intervention, a local anesthetic of xylocaine 2%** with 1:100,000 epinephrine was used in the area of surgical intervention.

Throughout the course of each surgical procedure, strict conditions of asepsis were maintained for the operator and the monkey's protection. All pertinent clinical observations were recorded.

B. GENERAL PREPARATION

Full mucoperiosteal flaps from the distal of the canine to the distal of the second molar were utilized for both the creation and the correction of the defects. An intrasulcular incision was carefully performed with a #15-C Bard-Parker blade and reflected with a small periosteal elevator(#7 wax spatula). Care was taken so thae tissue would not be perforated, and the incision was scalloped for better primary closure. Tissue tags on the

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*Parke, Davis and Co., Detroit, Michigan
**Astra Pharmaceutical Products Inc., Worchester, Mass.

inner surface of the flap were removed with a small tissue scissors. Gracey curettes were used to remove all interproximal granulation tissue and to thoroughly plane the exposed root surfaces.

C. PREPARATION OF THE DEFECTS

The sites chosen for the creation of the osseous defects were the interproximal osseous septae distal to the first premolar and second premolar. These areas offered a sufficient amount of bone present to allow for the creation of the two-walled defects, and these areas were easily accessible and readily visible. This experimental area also affords protection postoperatively due to the interproximal contacts between adjacent teeth, and due to the contour of the crowns. The control defect was placed distal to the second premolar, and the defect created distal to the first premolar received the culture medium implant.

Two-walled, surgically created, osseous defects were made in the selected sites. A #701 tapered fissure bur was used in a slow speed dental handpiece to remove 3 mm. of alveolar crest bone, along the distal root surface. The depth and width of the defect(3 mm.) was carefully

monitored with the use of a calibrated periodontometer.* The resulting osseous defects consisted of a lingual and distal wass of bone, a mesial wall of cementum and/or dentin and, no buccal wall(Figure 2). While removing the bone during the creation of the defects, isotonic saline was used as a coolant to avoid temperature increases which could damage the bone.

D. INTRODUCTION OF CHRONIC IRRITANTS

A wooden toothpick approximately 6 mm. in length was introduced into each osseous defect to serve as a chronic irritant, and the excess afforded a wedging effect for retention of the toothpick. The wood acted as a riute of direct communication from the oral cavity to the defect, allowing bacteria and their toxins ingress. This was performed in an attempt to simulate a chronic defect seen in human periodontitis.

The full mucoperiosteal buccal flap was repositioned and secured close to its original position with interrupted vertical matress, interproximal 5-0 Ethaflex**

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*Hu-Friedy--Michigan Probe
**Ethicon--Johnson and Johnson

sutures, and were tied on the lingual surface. This suturing technique provided additional stabilization of the chronic irritant.

E. POSTOPERATIVE CARE

The monkey received a prophylactic injection of an antibiotic, prior to the initiation of any surgical procedure. An intramuscular injection of 600,000 units (3 cc.) of Combiotic*(Penicillin and dihydrostreptomycin) was given. The Combiotic gives a 72 hour antibiotic coverage to the monkey. Postoperative instructions specified that the animals receive no food for the first 24 hours, a soft diet for the following 2 days, and a normal diet thereafter.

F. REMOVAL OF IRRITANTS

Seven days following the creation of the osseous defects, the animal was sedated, as before with Serylan, and the sutures and wooden irritants were removed. Clinically inflamed gingiva was noted at this time.

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*Pfizer Company

G. PROCUREMENT OF IMPLANT MATERIAL

Medium 199*(Appendix I), an instant tissue culture powder medium, was mixed with triple distilled water until a thick, pasty consistency was obtained. This culture medium paste without any other modification was utilized as the implant material.

H. CORRECTION OF THE DEFECTS

As outlined previously, the same surgical procedures were performed. Exactly 30 days postoperative to the creation of the two-walled osseous defects, the osseous defects were exposed for surgical implantation or curettage (in the case of the control). All chronic inflammatory tissue was carefully removed from the previously created two-walled osseous defects, and the root surfaces were thoroughly planed.

The control defect on the distal of the second premolar was carefully debrided and irrigated with isotonic saline, in order to leave the control defect free of any material.

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*Grand Island Biological Co., Grand Island, New York

The Medium 199 paste was placed in the debrided defect distal to the first premolar and overfilled. The implant material was placed easily, hemorrhage in the area was well controlled(Figure 3).

The control area and the implant area were covered by the full mucoperiosteal flap which was repositioned and held firmly in place with sutures(as previously described). The same postoperative antibiotic and diet instructions as previously described were given.

Seven days after the surgical correction of the osseous defects, the animal was again sedated for suture removal. The monkey was then maintained until the next procedure or until the scheduled sacrifice.

I. COLLECTION OF SPECIMENS

The animals were sedated as previously described and sacrificed by giving an intra-arterial injection of a lethal dose of Totaltox.*

A Stryker saw was used to obtain the block sections. The specimens were washed with distilled water and placed in pre-labeled jars containing 10% formalin for fixation.

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*Chicago Veterinary Supply, Chicago, Illinois

The volume of formalin was 20 times that of the specimen.

J. PREPARATION FOR HISTOLOGIC EXAMINATION

After two weeks of fixation, each specimen was decalcified in formic acid and sodium citrate (50%/50% solution), trimmed, embedded in paraffin, sectioned at 10 microns in a transverse buccolingual plane, and stained with hematoxylin and eosin. The degree of decalcification was determined by radiographs taken at weekly intervals after an initial four week period. The slides from each experimental site were stained with hematoxylin and eosin, and a representative histologic section was selected for a detailed histologic analysis.

CHAPTER IV

RESULTS

A. CLINICAL OBSERVATIONS

Both monkeys displayed a marginal chronic gingivitis at the beginning of the investigation(Figure 1). The papillary and marginal gingiva appeared mildly edematous and magenta in color. The attached gingiva was generally firm and pink in color. The sulcus depths were within acceptable limits. Seven days following the creation of the two-walled osseous defects and placement of the irritants the gingiva demonstrated an increased degree of inflammation. The marginal and papillary gingiva was edematous, erythematous, and displayed hemorrhage upon probing. Thirty days following the creation of the osseous defect, a decrease in the degree of gingival inflammation was noted as compared to seven days following the creation of the osseous defect, but greater than at the initiation of the investigation.

Additional bone resorption was detected in the defect areas at the re-entry 30 days after their creation. This additional resorption in the defect areas was noted

mesially and distally on the buccal cortical plate giving a larger V-shape to the defect.

Seven days following the correction of the defects, the marginal gingiva was edematous, boggy and erythematous as compared to adjacent areas. The tissue inflammation decreased by the fourteenth postoperative day and appeared close to normal.

B. HISTOLOGIC OBSERVATIONS

1. CONTROL

a. Epithelium

0 Day Figure 4.

Keratinized stratified squamous epithelium was present but not adherent to the tooth. A normal, thin sulcular epithelium was observed.

3 Day Figure 5.

The full mucoperiosteal flap over the defect area was probably lost in the histologic preparation, and therefore could not be evaluated. The sulcular epithelium exhibited some intercellular edema, some degeneration, and a fibrinopurulent exudate.

7 Day Figure 6.

Keratinized stratified squamous epithelium was present. Sulcular epithelium of normal thickness was noted.

14 Day Figure 7.

Keratinized stratified squamous epithelium

was present. The epithelial attachment was present(separated in histologic processing).

21 Day Figure 8.

The keratinized stratified squamous epithelium and the non-keratinized sulcular epithelium appeared normal.

28 Day Figure 10.

Keratinized stratified squamous epithelium was noted.

42 and 56 Day Figures 11 and 12.

A keratinized stratified squamous epithelium was noted. The "col" area epithelium was thin as normally expected.

b. Connective Tissue

0 Day Figure 4.

There was a plasmocytic infiltration and some edema. A few small spicules of bone were noted embedded within the collagen fibers adjacent to the defect. Hemorrhage was seen near

the detached flap.

3 Day Figure 5.

Loosely arranged tissue fibers were present. A fibrinopurulent exudate was present in the defect area and polymorphonuclear leukocytes were noted.

7 Day Figure 6.

The connective tissue showed some plasma cell infiltrate, proliferating fibroblasts, and young capillaries.

14 Day Figure 7.

Collagen, both mature and immature, was noted as the defect was approached. Inflammatory changes and cellular infiltrate, especially adjacent to the sulcular epithelium, were noted. A fibrous attachment is forming and new cementum is forming near the defect.

21 Day Figures 8 and 9.

The connective tissue repair by fibro-

genesis was generally completed.

28 Day Figure 10.

The lamina propria was composed of mature connective tissue. Some degree of inflammation was noted especially near the epithelium.

42 and 56 Day Figures 11 and 12.

The lamina propria shows mature collagen with some inflammatory changes of slight edema and some plasma cell infiltration. There is newly forming periodontal ligament.

c. Alveolar Bone and Defect

0 Day Figure 4.

The defect was noted in alveolar bone. Some small spicules of bone were noted among the fibrin clot debris.

3 Day Figure 5.

Alveolar bone apical to the created defect showed the periodontal ligament to be attached to the cemental surface of the root. The defect

was filled with a fibrinopurulent exudate, loosely arranged fibrous connective tissue, and polymorphonuclear leukocytes. Active osteogenesis with osteoblasts was seen within the marrow spaces adjacent to the defect area. Along non-defect bone, osteoblastic activity was noted.

7 Day Figure 6.

The defect area had many proliferating fibroblasts, young capillaries, and polymorphonuclear leukocytes. There was residual necrotic bone and new bone forming on the surface of old bone.

14 Day Figure 7.

Cementum formed on the root dentin surface adjacent to the defect, allowing for a reattachment. Within the defect there were proliferating and maturing fibroblasts with collagen fiber bundles being formed. Some new bone was forming in the defect, showing active osteoblastic activity.

21 Day Figures 8 and 9.

Some bone repair in the defect is noted with osteoblastic activity.

28 Day Figure 10.

A small amount of bone regeneration and osteoblastic activity was noted adjacent to the more mature bone. The section, as cut, reveals a new periodontal ligament fiber attachment to the newly forming cementum of the tooth.

42 and 56 Day Figures 11 and 12.

A small amount of new bone apposition is noted over the old alveolar bone with active osteogenesis. Young collagenous fibers extend from the new bone to newly formed cementum. A fibro-periosteum is noted over new bone.

2. MEDIUM 199 IMPLANT

a. Epithelium

0 Day Figure 13.

Just prior to sacrifice, the area was surgically flapped and curetted and Medium 199 implanted into the osseous defect. A keratinized stratified epithelium was present but not adherent to the tooth.

3 Day Figure 14.

Over the defect area the specimen did not show epithelium probably due to the histologic processing and existence of a dense fibrinopurulent exudate below making a weaker attachment to the underlying connective tissue.

7 Day Figure 15.

An ulcerated keratinized squamous epithelium was present showing intercellular edema. The "col" area epithelium was present, thin and not keratinized as expected.

14 Day Figure 16.

A keratinized stratified squamous epithelium was present.

21 Day Figure 17.

A keratinized stratified squamous epithelium was noted.

28 Day Figure 20.

A keratinized stratified squamous epithelium was present as was the sulcular epithelium.

42 Day Figure 22.

A keratinized stratified squamous epithelium was shown. The specimen was sectioned so as to not demonstrate the sulcular epithelium.

56 Day Figure 24.

The specimen shows a keratinized stratified squamous epithelium. The "col" area epithelium appears normal and non-keratinized.

b. Connective Tissue

0 Day Figure 13.

A plasmocytic infiltration and slight edema were noted. Hemorrhage was seen as were some spicules of bone adjacent to the defect.

3 Day Figure 14.

A fibrinopurulent exudate was present in the wound site. Many polymorphonuclear leukocytes and red blood cells were also noted. Some loosely arranged collagen fibers could be seen. Pigment granules were noted in the wound site which may represent particles of Culture Medium 199.

7 Day Figure 15.

This specimen showed many young capillaries and proliferating fibroblasts among collagenous fibers in the presence of a few inflammatory cells. This is the granulomatous reaction within the connective tissue. Active cementogenesis can be seen adjacent to an area of deformed tooth surface.

14 Day Figure 16.

This specimen demonstrates very active fibrogenesis, with abundant and dense collagen. Some minor inflammatory infiltrate is present in the superficial area. Basophilic particles, perhaps Culture Medium 199, are evident in this slide as well.

21 Day Figure 17.

Some minor plasma cell infiltrate and edema are present in the lamina propria(normal). Periodontal ligament fibers are seen in contact with bone and cementum and some appear to lead to the fibrous connective tissue and into the gingiva demonstrating that the periodontal ligament apparatus has reformed.

28, 42 and 56 Day Figures 20, 22 and 24.

Even less plasma cell infiltrate is evident in the lamina propria. The connective tissue displays dense fibrous repair. The periodontal ligament appears cellular and fibers have a functional orientation. In the

56 day there appears to be an accidental surgical defect in the tooth surface, new cementum having repaired over it allowing for a reattachment of the ligament fibers.

c. Bone

0 Day Figure 13.

The well formed, rectangular, surgically created defect can be clearly seen. A fibrin clot, debris, red blood cells, some connective tissue and bone spicules could be seen in this defect.

3 Day Figure 14.

Red blood cells, polymorphonuclear leukocytes and a fibrinopurulent exudate are seen in the defect area. Active osteogenesis is seen in the marrow areas adjacent to the defect area as well as some osteoclastic activity. Pigment granules, perhaps Culture Medium 199, are evident in the specimen.

7 Day Figure 15.

On the surface of the bone there is evidence of osteoclasia and reactive apposition of new bone. In the marrow spaces Howship's lacunae with osteoclastic activity and increased proliferation of cells were seen involved in remodelling.

14 Day Figure 16.

Small spicules and islands of bone with osteoblastic activity demonstrated new bone formation. The pigmented granules evident in earlier sections was seen in this specimen also. Osteoclasts were seen in the marrow spaces.

21 Day Figures 17 and 18.

Crestal bone demonstrated spicules of newly formed bone with young osteoblasts surrounding them and apposition occurring. In the marrow spaces osteoblastic activity is present and repair was occurring here as opposed to osteoclastic activity seen in previous specimens. The basophilic granular material is evident in this specimen also.

28 Day Figure 20 and 21.

Cortical bone shows evidence of new bone formation and reversal lines. New bone at the crest is evident with a mature and cellular marrow. Many bone spicules and islands are lined by osteoblasts and very active osteogenesis is occurring.

42 Day Figures 22 and 23.

On the surface of the cortical bone osteophytic bone formation is present. In this tangential section much immature newly formed bone is present, as well as abundant osteoblasts and active osteogenesis. Between the new bone and the tooth newly forming periodontal ligament fibers can be seen. This specimen demonstrated the peak osteoblastic activity in the experimental sections and the maximum amount of defect regeneration.

56 Day Figures 24 and 25.

New bone formation and normal periosteum are present at the cortical surface. Marrow

spaces ar filled with normal blood vessels and moderate to light cellularity. In the crestal area there is evidence of prior bone resorption (reversal lines) and apposition trapping many osteocytesl There appears to be a functional orientation of the periodontal ligament fibers between root cementum and new alveolar crest bone.

CHAPTER V

DISCUSSION

This investigation demonstrated that the implant material utilized, Medium 199, is well tolerated by the host. This material was successfully implanted in osseous defects of the rhesus monkeys with no rejection phenomena observed in the host. Two-walled osseous defects in the rhesus monkey showed repair by regeneration and reattachment following implantation with Medium 199; however, the control also demonstrated repair activity. In the Medium 199 specimens studied there appeared, on a quantitative basis, slightly more bone regeneration and osteoblastic activity than was seen in the control specimens.

The sequential healing description of gingival epithelium demonstrated its potential for swift and extensive repair. Rapid regeneration and repair were also seen within the connective tissue of the mucoperiosteal flap. Dominant cellular activity was demonstrated by fibroblasts and endothelial cells.

To maintain an environment rich in nutritive materials is of primary concern with this thesis. An environment rich in the materials necessary to the

maintenance and growth of tissue is provided by culture medium. A local environment for cell support and regeneration is established in chamber diffusion techniques, and established in "bone induction properties". A material such as tissue culture medium, used as an implant, does not provide cells themselves to re-establish bone in an osseous defect, but may provide the "life line" to successful proliferation of osteoid material and bone formation. One may not need an actual bone cell which in transplant experiments actually is noted to frequently resorb and be replaced. But if a material that is present can support the life of bone and induce growth of existing bone, then osteogenic potential of a graft material can be most favorable.

As with normal nutrition, required nutrients are delivered to the cell area via the circulation with required nutrients being utilized by the cell for cell regenerative processed and tissue regeneration. The literature review stated that providing certain minerals, vitamins, amino acids, salts and other substances can support cell life, maintenance, and regeneration. In fact, it was noted that factors normally present in whole serum can be replaced by a synthetic mixture. Addition of

certain required essential amino acids, vitamins, serum protein and minerals can provide survival of mammalian cells for long periods of time. Perhaps, the addition of these essential nutrients via Medium 199 into osseous defects can explain the appearance of increased bone regeneration and osteoblastic activity in the experimental specimens.

Culture medium provides the essential nutrients of whole serum and in the results of the experiment was well tolerated without a remarkable inflammatory condition resulting and without rejection. The fact that this material was well tolerated by the tissues is of significant interest. That bone formation was noted in the subject sections is consistent with the literature showing this material may be capable of providing an environment of bone cell support and production.

In reviewing the control to subject histologic sections it was noted that control slides also showed some bone regeneration. This is not an inconsistent finding since some repair is a normal healing response.

Osteogenesis and how it is induced are of prime importance in this and future periodontal research. Bone induction was discussed by Urist[33] who termed the

"bone induction principle" stating that a tissue substrate may exert a physio-chemical effect upon competent mesenchymal cells to stimulate their differentiation into osteoblasts capable of both osteogenesis and further induction(autoinduction). Thus, local cell environment may be a determining factor in bone maintenance and growth.

Osteoblasts have been suggested to arise from three possible origins: Burns[35] suggested that they arise from pre-existing osteoblasts; Ham and Harris[36] suggested that they arise from the endosteal lining of the marrow cavities; and Bloom[37] suggested that they arise from the previous two sources plus from the perivascular undifferentiated mesenchymal cells. These three sources can act simultaneously.[37] The initiating factor in bone formation has not been isolated yet. Culture medium was implanted because of its potential to provide the raw building blocks in animal bodies. Maintenance, support, and regeneration of bone is this paper's concern on a local, cellular level. We have already noted by the many <u>in vitro</u> culture media studies, that creating a desirable environment can result in bone cell support and in actual osteogenesis. Also, we have established by the

chamber diffusion studies and cited in the literature "bone induction properties", that in a local environment a condition can be created in bone for cell support and regeneration. The possibility, therefore, of this material being an inductor for osteogenesis exists.

The implant material in occupying the surgical defect may provide not only nutrients to bone regeneration but also a space for its formation free of the usual characteristic clot formation that recurs in such a defect and results after with fibrous repair. It was demonstrated that culture media is a compatible implant material. any compatible implant material may occupy space and delay healing of a defect. This would allow a greater proliferation of granulation tissue with its increased numbers of undifferentiated mesenchymal cells when compared to control defects. A greater potential for osteogenesis may thus be established since an increased pool of potential progenitors of osteoblasts is present. Procedures which utilized sclera[83] as an implant graft material persisted and active osteogenesis was noted at the graft's edge during periodic re-entry for a two year period, which may represent the sclera graft being transformed into new bone. Thus, the scleral graft may hve just occupied space and allowed host

site cells to become competent of induction themselves. The culture medium may act as a "space occupier" in this manner.

There was a small basophilic particulate material present in the experimental sections at 3, 14 and 21 days that did not appear at all in the control sections. Most probably this might represent Medium 199. These particles demonstrated no rejection phenomenon surrounding them and, if Medium 199, these particles might account for the increased bone regeneration and osteoblastic activity in experimental sections not seen to such a degree in the control.

Also of interest is that the culture medium did not interfer with the formation of either a periodontal ligament or cementum. This is of primary significance in healing of successful periodontal osseous procedures as bone is only a part of the triad of periodontal support. Reconstruction of new cementum, periodontal ligament and supporting alveolar bone must be achieved simultaneously, and culture medium did not interfer with this formation of a total new attachment apparatus.

It must be kept in mind that the complex and often unknown etiologic factors of periodontal disease in humans

are not paralleled by simulation in a model such as the rhesus monkey. Therefore, healing occurred without the influence of many complicating factors. But it is significant that culture medium may help induce bone formation or at least is compatible with tissue regeneration and repair. Since no model can exactly simulate the disease entity as it exists in may except man, only studies in man will offer valid and sufficient insight into repair and regeneration of diseased periodontal structures.

Surgical procedures to support or provide an environment conducive to the re-establishment of osseous tissue is, obviously, the most desirable methodology to be sought in periodontics. Unfortunately, the degree of predictability is often quite unstable for present surgical regenerative procedures. It would be ideal to find a biologically acceptable material, free of communicability of disease, without rejection phenomenon, or an immunologic reactive capacity.

It has been well established that culture medium has supported life and growth of osseous tissues *in vitro*; and limited available research, as previously noted, showed similar findings *in vivo*. This is the first use of a culture medium in the periodontal defect. It would be

wise to test varying concentrations of culture medium. There are many media, varying in constituents and relative concentrations of constituents that are used for different purposes in in vitro studies. Further research could investigate whether a medium other than Medium 199 would be even more compatible with cell and osseous regeneration in periodontal defects. Also to possibly incorporate cancellous osseous implants combined with culture medium as an osseous-alloplast graft material may be investigated. Results of this study definitely support the value of further investigation.

CHAPTER VI

CONCLUSIONS

1. The two-walled osseous defects corrected by Medium 199 were accepted by the host and did not impede osteogenesis.

2. Medium 199 may act as a "space occupier" which allows for a greater number of undifferentiated mesenchymal cells and host induction of osteogenesis.

3. The experimental defects created in this study attempted to simulate chronic human periodontal disease. However, this experimental model may not allow for a direct relationship to the healing response in humans.

4. Some degree of repair is expected in both the control and experimental specimens. There did consistently appear to be a greater degree of osteoblastic activity, osteogenesis and new bone regeneration in the experimental sections when subjectively compared to the control sections; although, an exact statistical analysis becomes impossible due to the slide preparation and sectioning procedures utilized in this experiment.

5. In view of the results of this study, further investigation into this and other culture media and the mixture of a medium and other implant materials including osseous material may prove beneficial in the treatment of periodontal osseous defects.

CHAPTER VII

SUMMARY

The purpose of this investigation was to examine histologically the sequential healing phenomonen of surgically created two-walled osseous defects in rhesus monkeys using Medium 199 as an alloplastic implant material.

Two male adult rhesus monkeys were utilized as experimental models providing specimens from 0 to 56 days postoperatively. Both control and implant specimens were taken at 0, 3, 7, 14, 21, 28, 42, and 56 days. The control surgical defects were treated by surgical curettage only.

Commercially available Medium 199 was mixed with triple destilled water until a thick paste consistency was obtained and was utilized as the experimental implant into the surgically created defects.

Histologic sections from each specimen were stained with hematoxylin and eosin, a description of the sequential healing events was recorded and the results discussed.

The implanted Medium 199 was compatible with osteogenesis. The material was well accepted by the host site

and did not impede osteogenesis. By a subjective comparison, it appeared, histologically, as though more bone regeneration and osteoblastic activity was seen in the Medium 199 specimens than was seen in the control specimens.

CHAPTER VIII

ILLUSTRATIONS

Figure 1. The preoperative appearance of the experimental area. Note the slight papillary and marginal gingivitis.

Figure 2. Experimentally created two-walled osseous defects(d) distal to the first premolar(implant) and the second premolar(control).

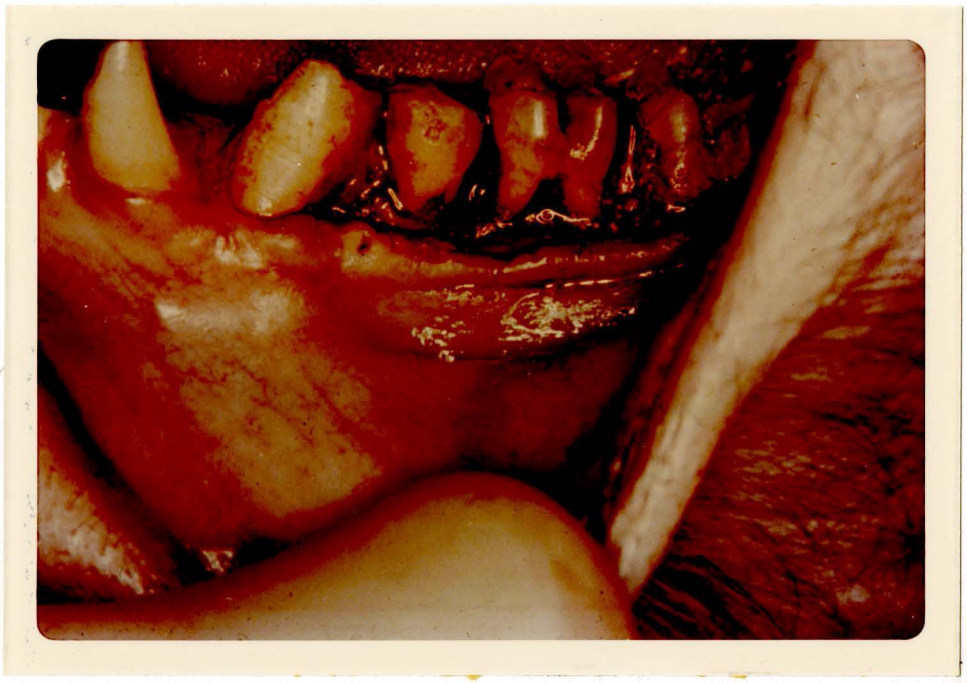

Figure 3. Placement of Medium 199 implant(overfilled) in the defect area distal to the first premolar and the control area is distal to the second premolar.

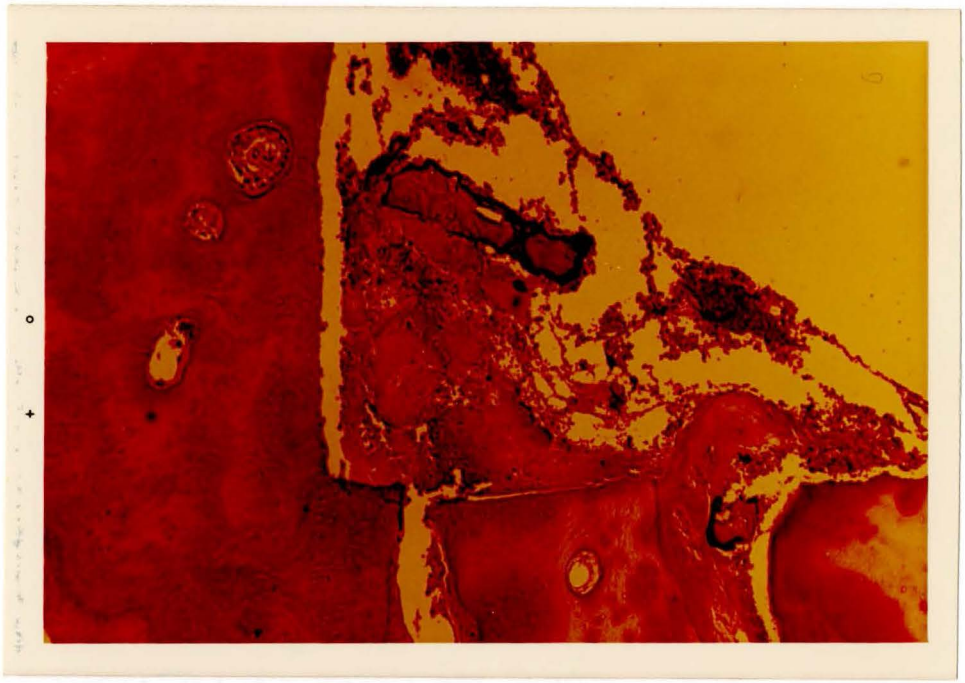

Figure 4. 0 day flapped control, 25x.
Surgically created defect with fibrin clot debris showing pieces of connective tissue and spicules of bone.

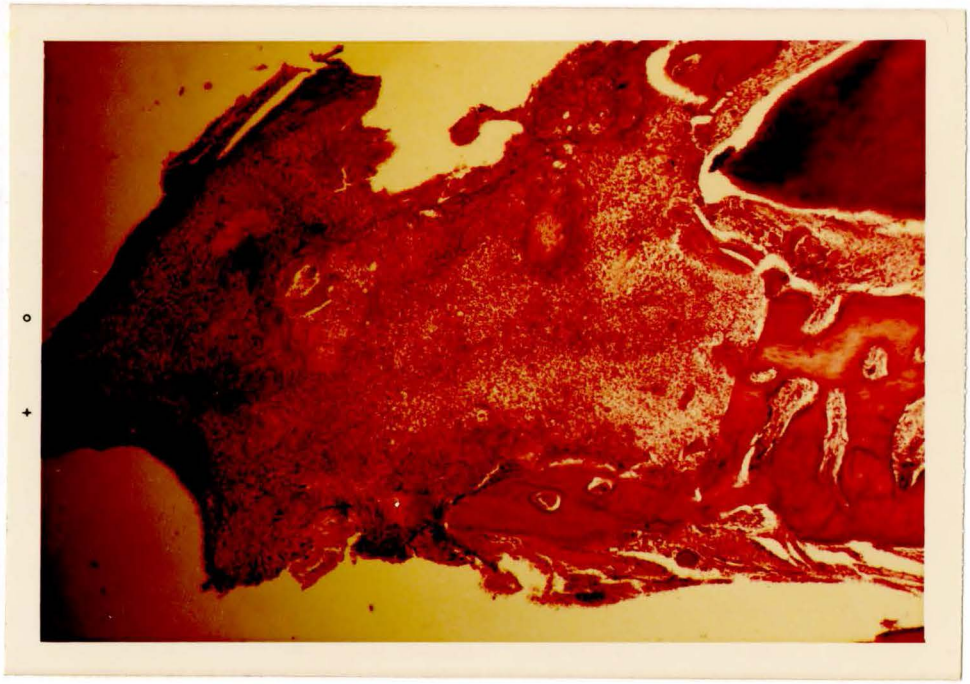

Figure 5. 3 day control, 25x.
The defect area is filled with a fibrinopurulent exudate.

Figure 6. 7 day control, 25x.

The defect has many proliferating fibroblasts.

New bone(n) is forming over mature bone(m).

Figure 7. 14 day control, 25x.
Stratified squamous epithelium(e), and new collagen(c) as defect is approached.

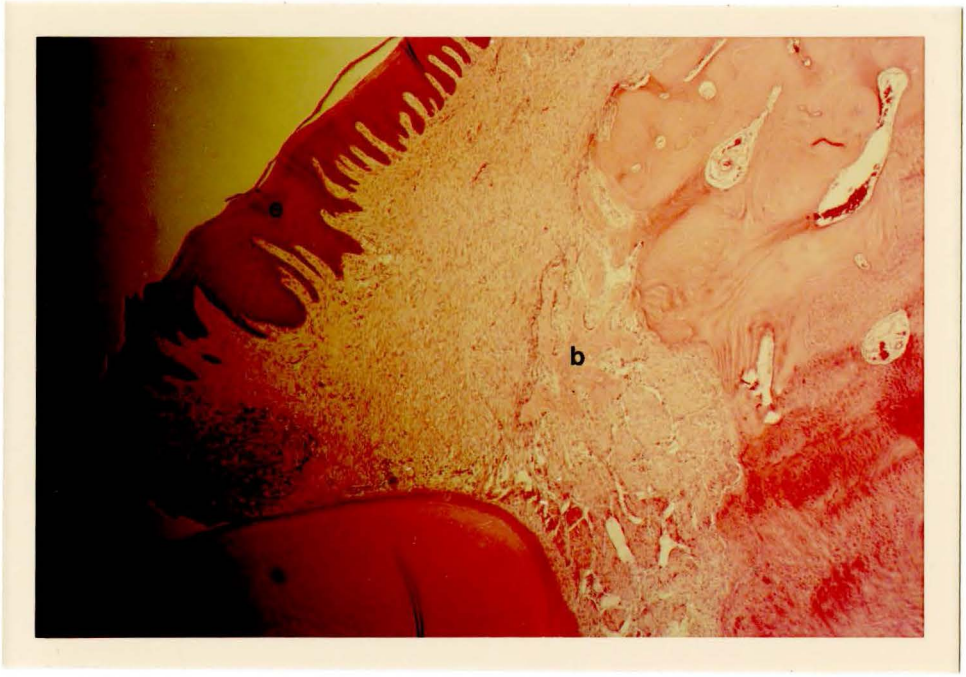

Figure 8. 21 day control, 25x.
Keratinized stratified squamous epithelium(e) and some new bone repair(b).

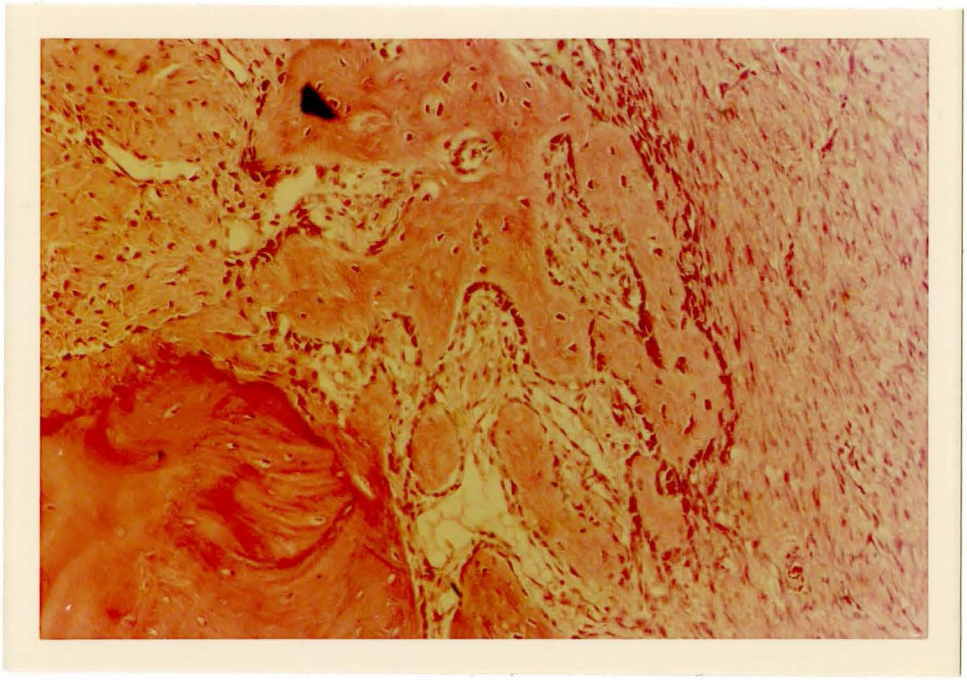

Figure 9. 21 day control, 100x.

Active osteoblastic activity.

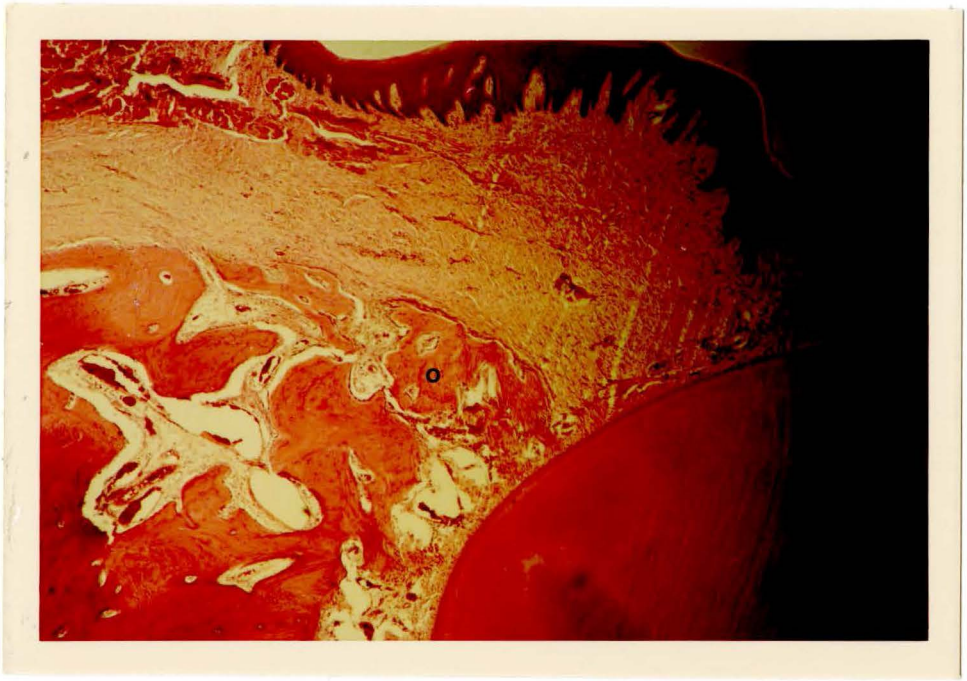

Figure 10. 28 day control, 25x.

Osteoblastic activity(o) adjacent to the more mature bone.

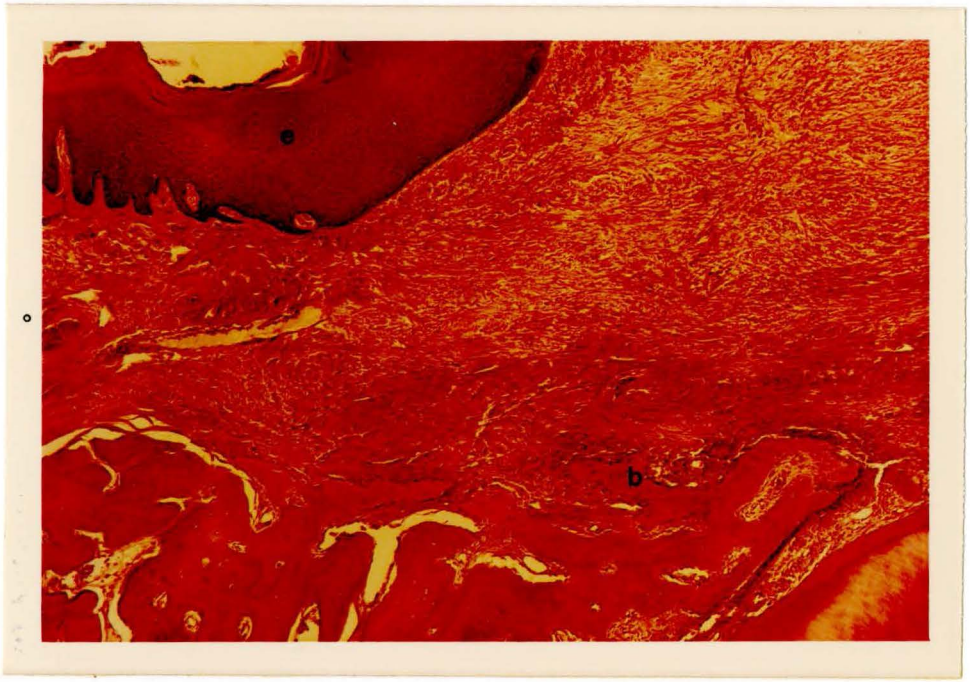

Figure 11. 42 day control, 25x.
Keratinized stratified squamous epithelium(e) and new bone formation(b).

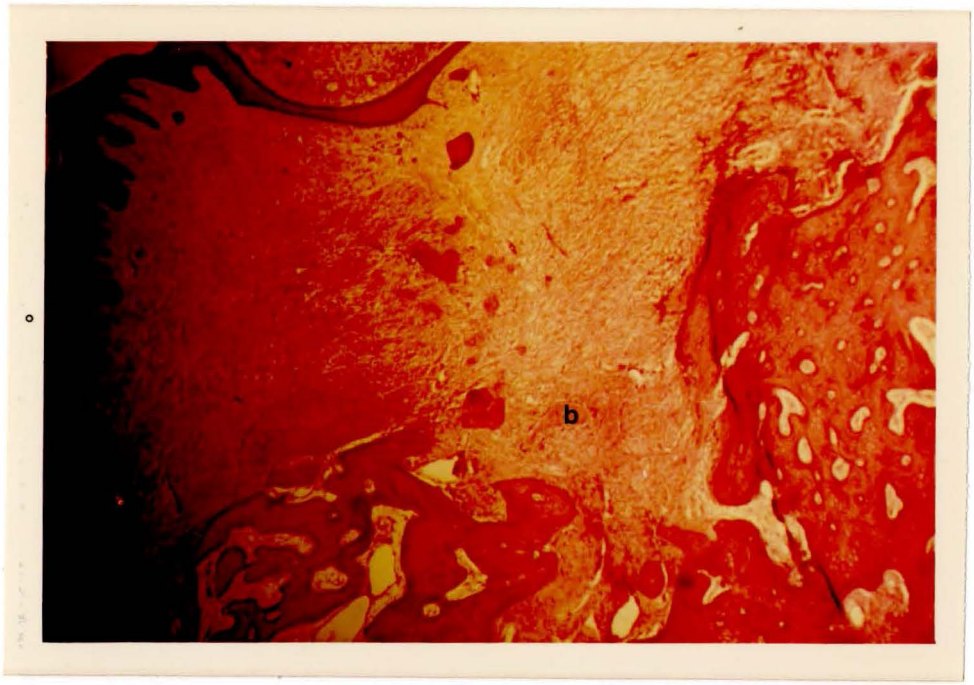

Figure 12. 56 day control, 25x.
Defect area with some new bone(b), a fibroperiosteum is noted over the new bone.

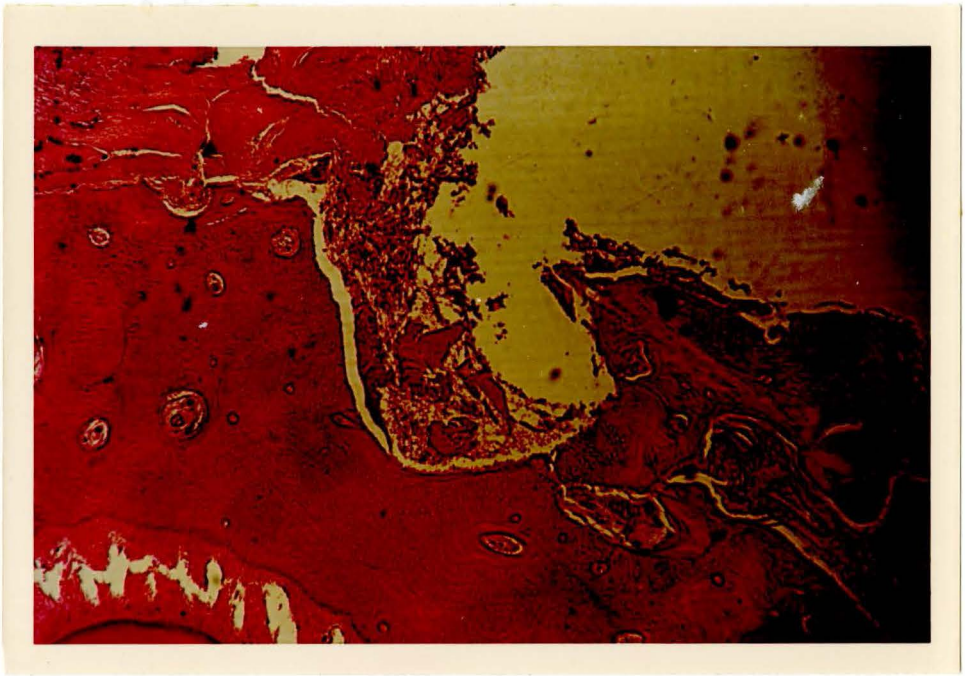

Figure 13. 0 day Medium 199 implant, 40x.
Surgically created defect with red blood cells, some connective tissue and bone spicules.

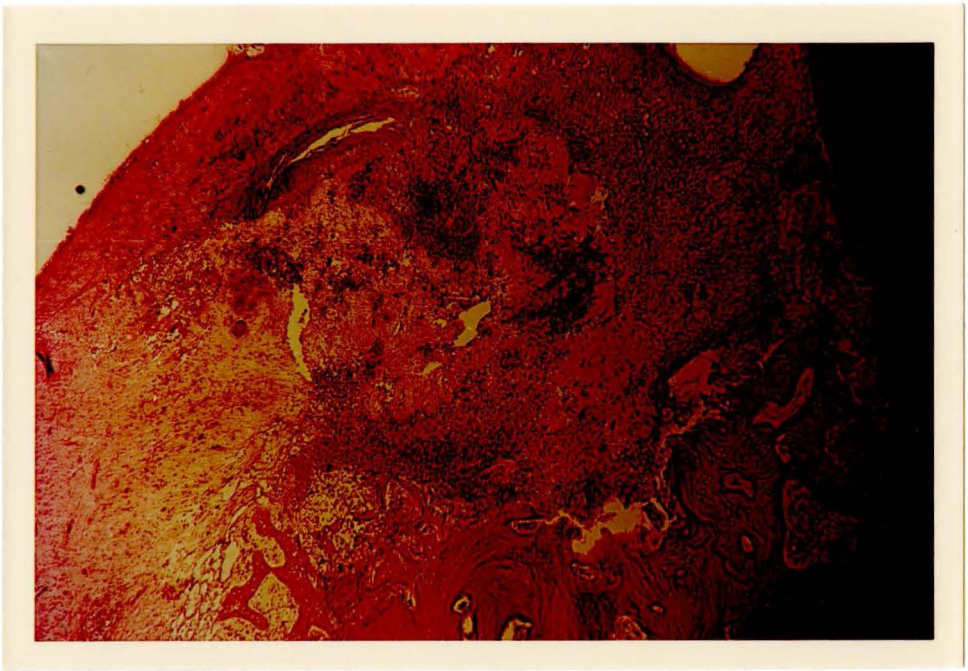

Figure 14. 3 day Medium 199 implant, 40x.
Red blood cells, polymorphonuclear leukocytes and fibrinopurulent exudate(e) are seen in the defect area.

Figure 15. 7 day Medium 199 implant, 40x.
Young capillaries and proliferating fibroblasts.
Active cementogenesis(c) can be seen adjacent to the tooth.
Evidence of osteoclasia and reactive apposition of new bone
(b) are seen.

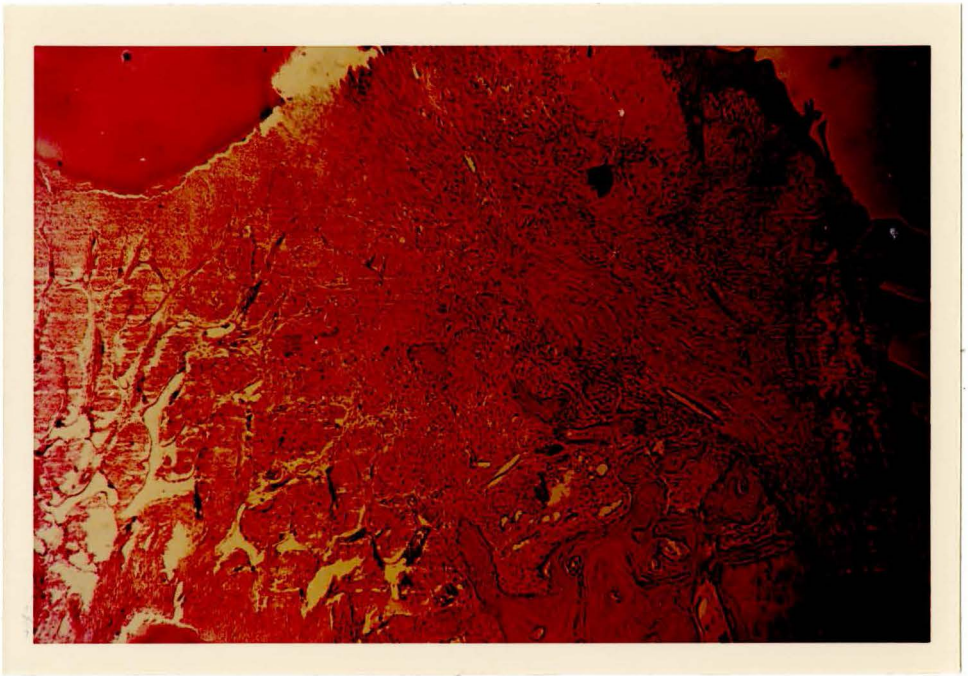

Figure 16. 14 day Medium 199 implant, 40x.
Active fibrinogenesis as well as spicules and islands of newly forming bone(s).

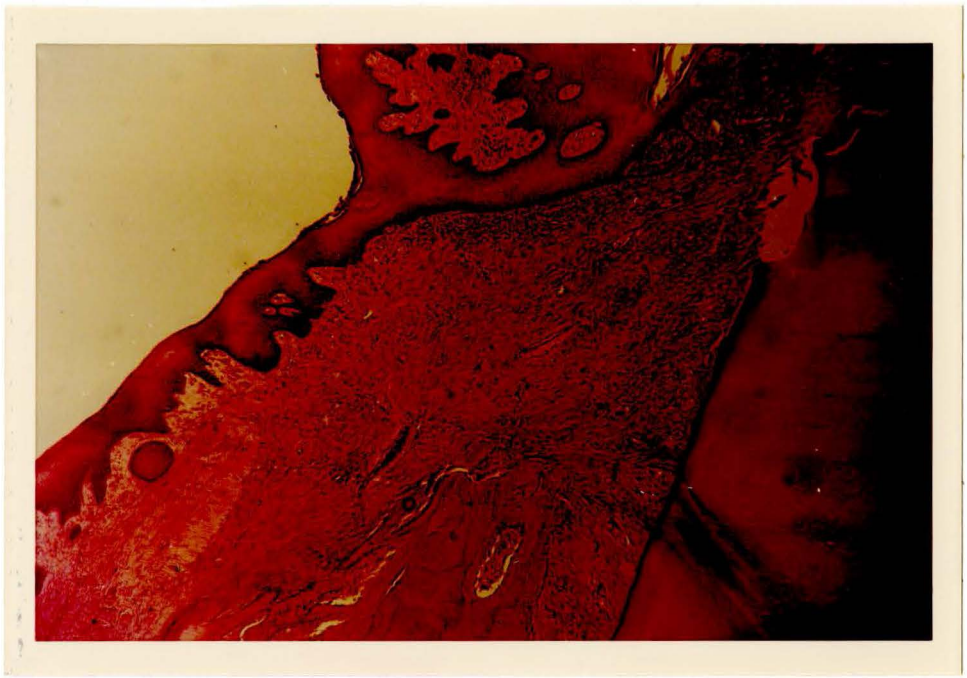

Figure 17. 21 day Medium 199 implant, 40x. Periodontal ligament fibers(f) and active crestal osteogenesis(o).

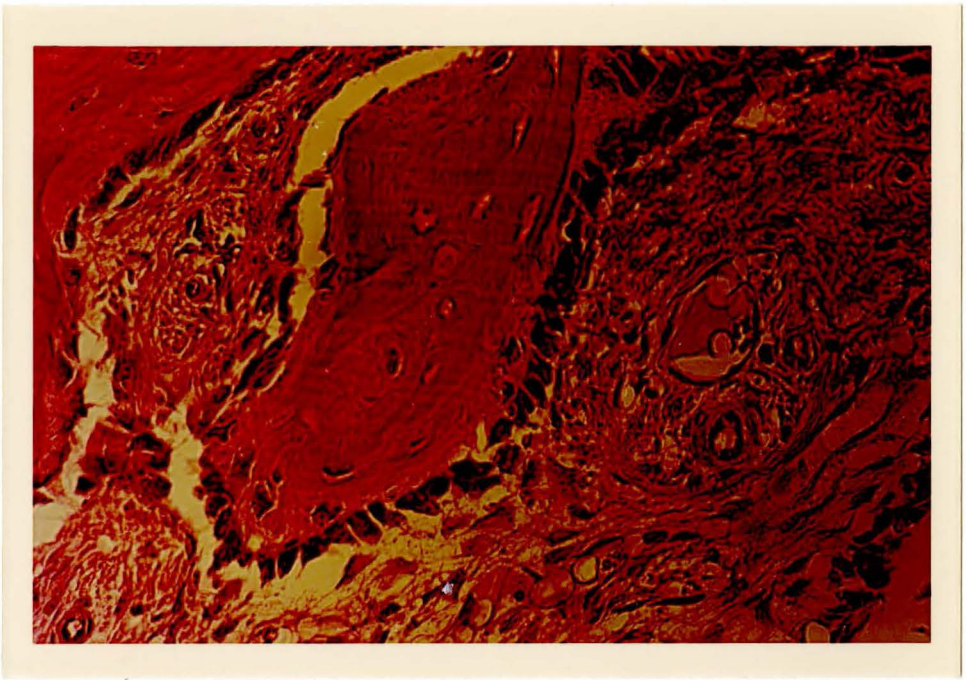

Figure 18. 21 day Medium 199 implant, 100x.
Active osteogenesis seen around a spicule of new bone.

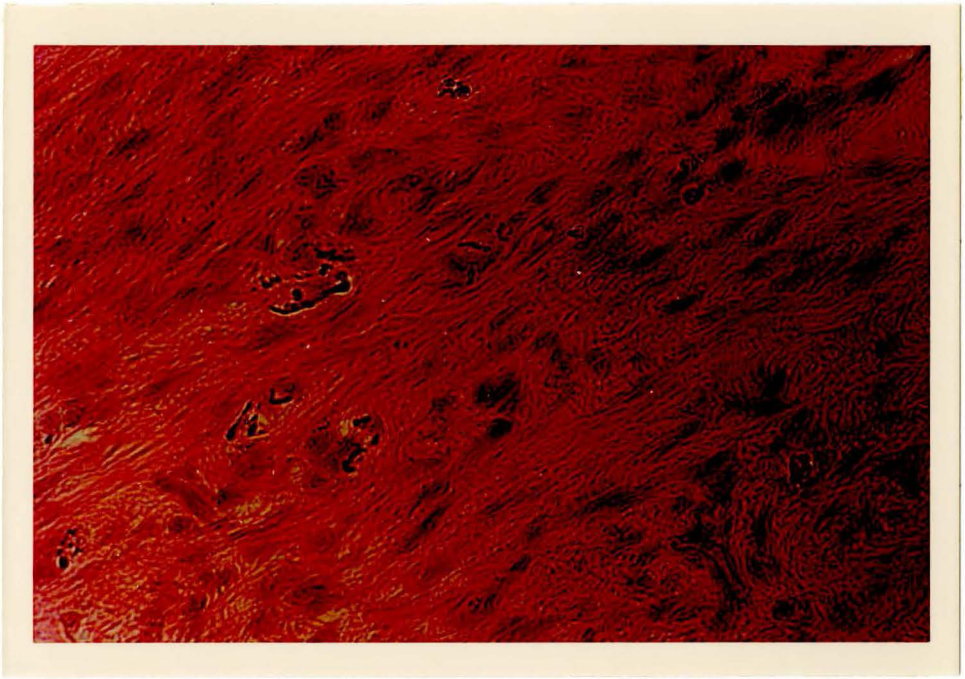

Figure 19. 21 day Medium 199 implant, 100x.
Basophilic particulate material.

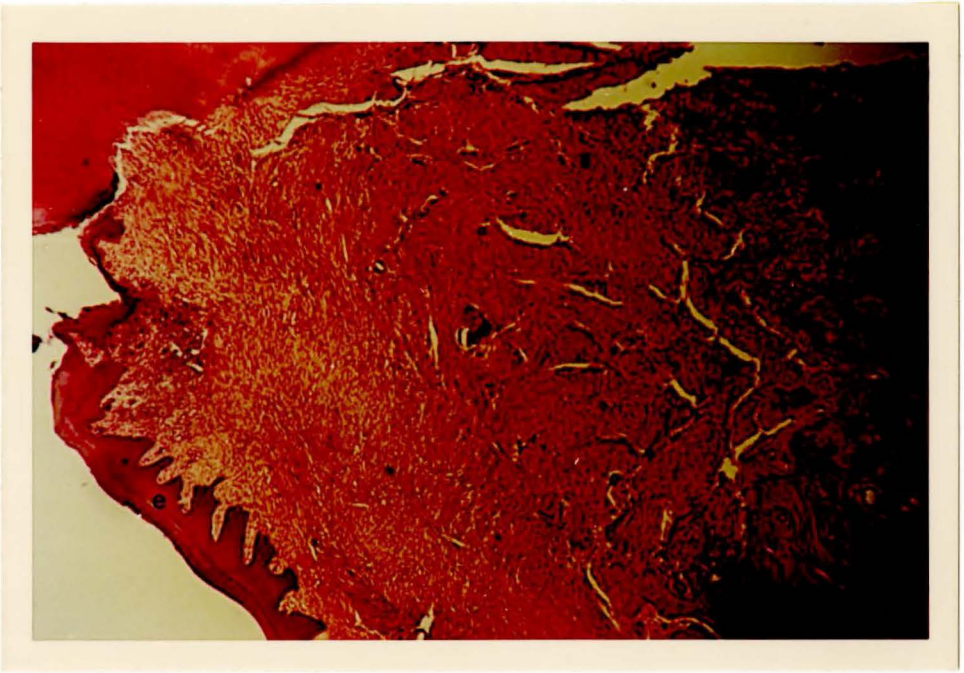

Figure 20. 28 day Medium 199 implant, 40x. Stratified squamous epithelium(e) and dense fibrous repair of the connective tissue.

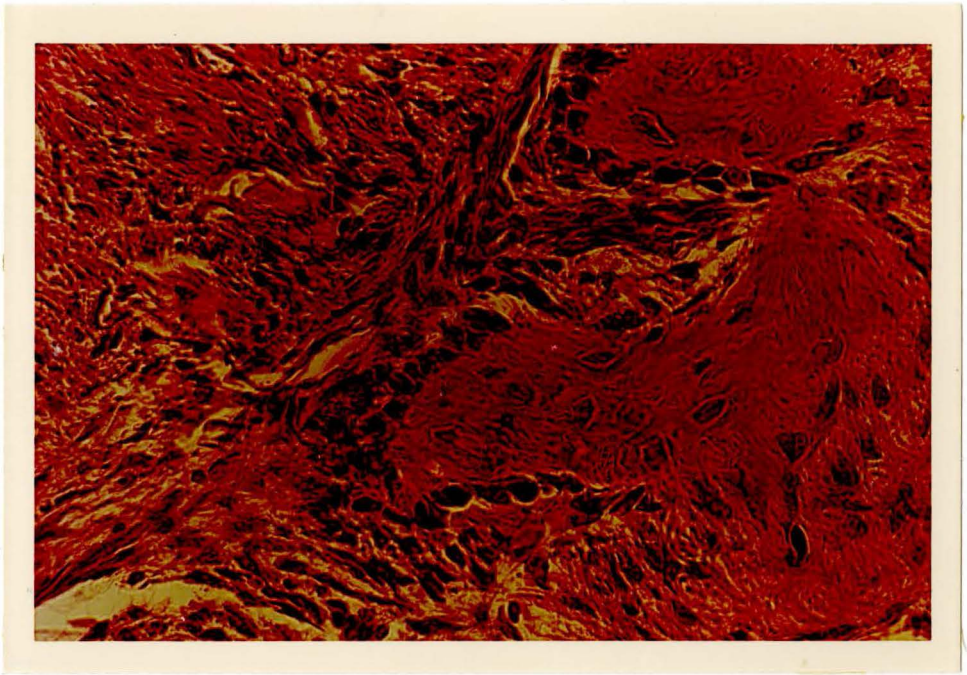

Figure 21. 28 day Medium 199 implant, 100x.
Osteoblasts lining a spicule of new bone(n).

Figure 22. 42 day Medium 199 implant, 40x.
Active new bone formation(n).

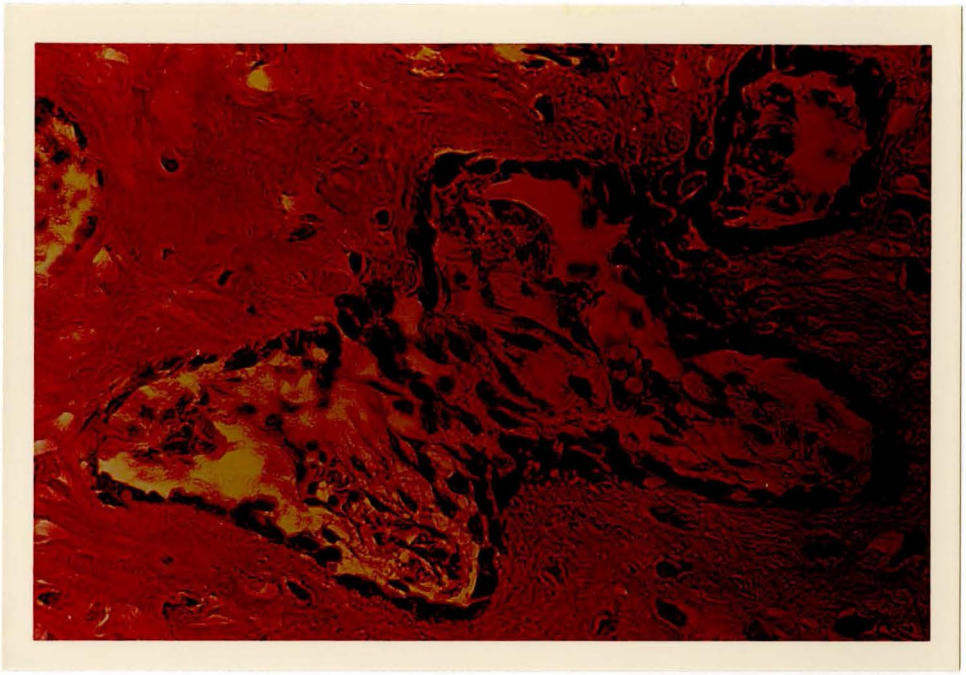

Figure 23. 42 day Medium 199 implant, 100x.
Marrow space showing active osteoblastic activity.

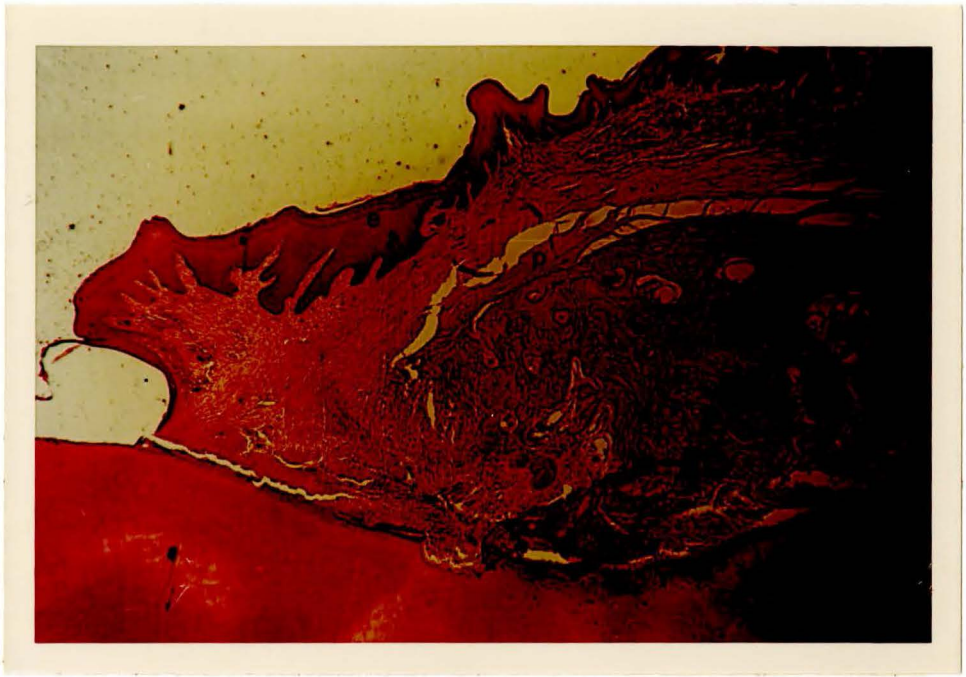

Figure 24. 56 day Medium 199 implant, 25x. Keratinized stratified squamous epithelium(e) and sulcular epithelium. Osseous repair with newly formed periosteum(p).

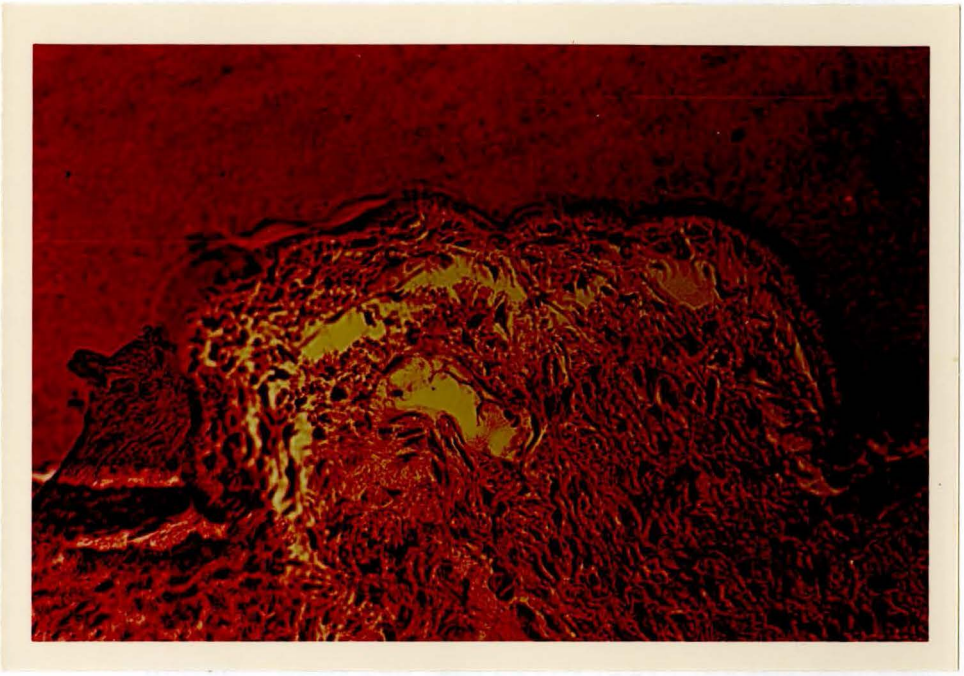

Figure 25. 56 day Medium 199 implant, 100x.
Accidental surgical defect showing cementogenesis(c).

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APPENDIX I

MEDIUM No. 199

L-Arginine	70.0	Riboflavin	.01
L-Histidine	20.0	Pyridoxine	.025
L-Lysine	70.0	Pyridoxal	.025
L-Tyrosine	40.0	Niacin	.025
DL-Tryptophan	20.0	Neacinamide	.025
DL-Phenylalanine	50.0	Pantothenate	.01
L-Cystine	20.0	Biotin	.01
DL-Methionine	30.0	Folic acid	.01
DL-Serine	50.0	Choline	.5
DL-Threonine	60.0	Inositol	.05
DL-Leucine	120.0	p-Aminobenzoic acid	.05
DL-Isoleucine	40.0	Vitamin A	.1
DL-Valine	50.0	Calciferol(D)	.1
DL-Glutamic acid	150.0	Menadione(K)	.1
DL-Aspartic acid	60.0	a-Tocopherol phosphate(E)	.01
DL-Alinine	50.0	Ascorbic acid	.05
L-Proline	40.0	Glutathione	.05
L-Hydroxyproline	10.0	Cholesterol	.2
Glycine	50.0	Tween 80(oleic acid)	20.0
Cysteine	.1	Sodium acetate	50.0
Adenine	10.0	L-Glutamine	100.0
Guanine	.3	Adenosine triphosphate	10.0
Xanthine	.3	Adenylic acid	.2
Hypoxanthine	.3	Ferric nitrate	.1
Thymine	.3	Ribose	.5
Uracil	.3	Deoxyribose	.5
Thiamin	.01		

This medium also contains a balanced salt solution(Hanks). The above values are milligrams per 1000 ml.

HANKS SALT SOLUTION
(Grams per litre)

NaCl	8.0	$Na_2HPO_4-2H_2O$	.06
KCl	.4	KH_2PO_4	.06
$CaCl_2$	.14	Glucose	1.0
$MgSO_4-7H_2O$	.1	Phenol Red	.02
$MgCl_2-6H_2O$	.1		

Taken from: Paul, J.: Cell and Tissue Culture. Livingstone Ltd., London, 1959. pp. 68&74.

APPENDIX II

Nutrients	Amount per day	
Water	2.3	liters
Energy	2.06	kcal
Amino acids	70	g
Glucose	213	g
Fat	106	g
Sodium	50	mmol
Potassium	50	mmol
Calcium	7.5	mmol
Magnesium	3.0	mmol
Iron	50	umol
Zinc	20	umol
Manganese	40	umol
Copper	5	umol
Chlorine	68.3	mmol
Phosphorus	13.5	mmol
Fluorine	50	umol
Iodine	1	umol
Thiamine	1.2	mg
Riboflavin	1.8	mg
Niacin	10	mg
Vitamin B_6	2	mg
Folic acid	.2	mg
Vitamin B_{12}	2	ug
Pantothenic acid	10	mg
Biotin	.3	mg
Ascorbic acid	30	mg
Vitamin A	.75	mg
Vitamin D	3	ug
Vitamin K	.15	mg
Tocopherol	100	mg

This is a complete intravenous nutrition for adults as given in: Ghadimi, H.: Total Parenteral Nutrition. John Wiley and Sons, Inc., 1975.

APPENDIX III

GRAFT TERMINOLOGY

Old	New	Definition
Autograft	Autograft	same individual
Isograft	Synograft	identical twins
Homograft	Allograft	within same species
Heterograft	Xenograft	different species
	Alloplast	bone substitute

APPROVAL SHEET

The thesis submetted by Peter Haupers, Jr., B.S., D.D.S. has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

4-18-78
Date

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